ACS SyntheticBiology

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Engineering *Escherichia coli* for Light-Activated Cytolysis of Mammalian Cells

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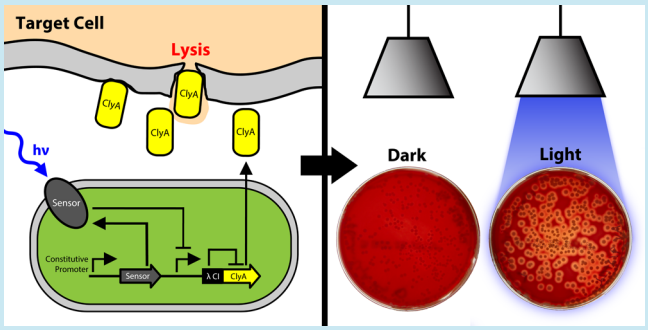
Supporting Information

ABSTRACT: By delivering payloads in response to specific exogenous stimuli, smart bacterial therapeutics have the potential to overcome many limitations of conventional therapies, including poor targeting specificity and dosage control in current cancer treatments. Although not yet explored as a trigger for bacterial drug delivery, light is an ideal induction mechanism because it offers fine spatiotemporal control and is easily and safely administered. Using recent advances in optogenetics, we have engineered two strains of *Escherichia coli* to secrete a potent mammalian cytotoxin in response to blue or red light. The tools in this study demonstrate the initial feasibility of light-activated bacterial therapeutics for applications such as tumor cytolysis, and their modular nature should enable simple substitution of other payloads of interest.



Synthetic biology has the potential to create novel therapeutic strategies to improve the treatment of diseases such as cancer. Conventional cancer treatment regimens involving chemotherapy, radiotherapy, or surgical excision are often limited by low targeting specificity, inadequate dosage control, or damage to healthy tissue, but these obstacles could be overcome by the development of 'smart' living therapeutics through synthetic biology approaches.[1] In particular, bacteria hold promise as potential therapeutic agents for cancer due to the relative ease of engineering genetic circuitry within them, the innate propensity of certain strains to target tumors, and their ability to actively penetrate solid tumors, as opposed to drugs that rely on passive diffusion.[1,2] Bacteria have been engineered with genetic circuits that allow for tumor cell invasion or therapeutic delivery in response to stimuli such as L-arabinose, hypoxia, cell density, γ-irradiation, and acetylsalicylic acid.[2]

In contrast to these stimuli, light can enable safe triggering of gene expression with fine spatiotemporal control without reliance on passive diffusion. Fiber optic systems enabling targeted and accurate light delivery into tumors are already used for photodynamic therapy.[3] Optogenetic control of drug delivery has been demonstrated in mammalian cells with blue light,[4] but this approach has not yet been demonstrated in bacteria or extended to light inputs of different wavelengths. In this study, we exploited bacterial optogenetic components to enable light-activated delivery of a cytolytic payload using *E. coli*. We achieved blue light-mediated cytolysis of mammalian cells with a common laboratory strain of *E. coli* and replicated these findings with a minimally immunogenic strain that is being explored for clinical applications. Since the longer wavelength of red light makes it more advantageous for therapeutic applications of optogenetics, we also constructed a plasmid (pMars) that allows for red light-controlled expression of a gene of interest and successfully utilized it to control cytolysis. Our results provide proof of concept that optogenetic control of cytotoxicity can be achieved in bacteria with multiple wavelengths of light.

RESULTS AND DISCUSSION

We selected the pore-forming toxin cytolysin A (ClyA) as the payload for our system because it is readily secreted *via* outer membrane vesicles[5] and it has been shown to reduce tumor growth when expressed by bacteria *in vivo*.[6] To characterize this toxin, we first codon optimized and synthesized the *clyA* gene for expression in *E. coli* and this gene was cloned into the

Special Issue: iGEM 2013

Received: October 24, 2013
Published: December 27, 2013

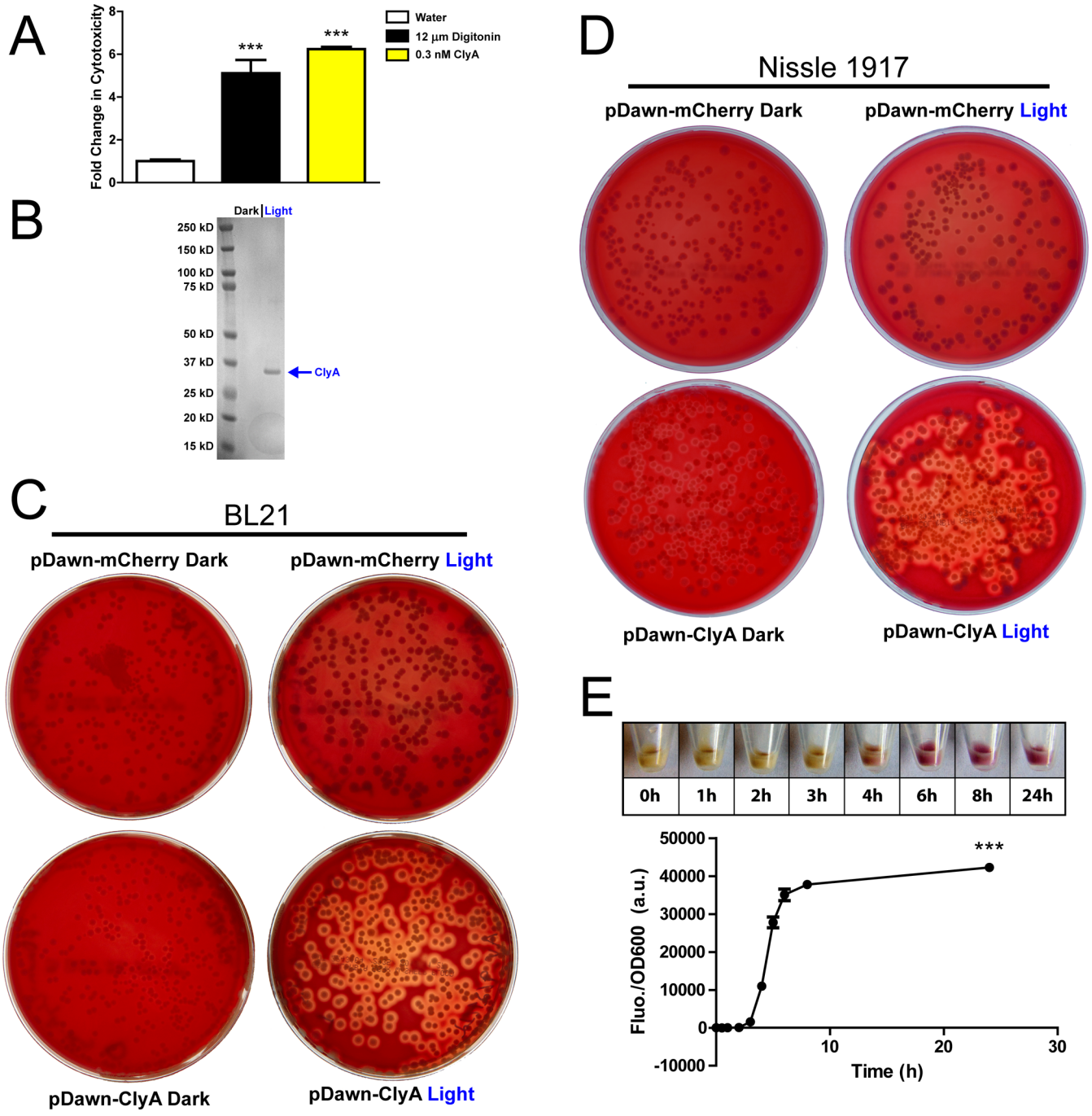


Figure 1. Blue light-activated cytolysis in *E. coli* BL21 and Nissle 1917 using pDawn. (A) Synthesized and purified ClyA lyses SKBR3 cancer cells *in vitro*. Purified ClyA (0.3 nM) resulted in a 6-fold increase in cytotoxicity, significant ($p < 0.001$) over both the negative control vehicle (water) and the positive control (digitonin, 12 μM) ($n = 6$ samples per group). (B) BL21(pDawn-ClyA) shows specific, blue light-dependent expression of the ~34 kDa ClyA protein. (C) In contrast to BL21(pDawn-mCherry), BL21(pDawn-ClyA) exhibits blue light-dependent cytolysis on blood agar. (D) Blue light-dependent cytolysis on blood agar is seen from Nissle 1917(pDawn-ClyA) but not from Nissle 1917(pDawn-mCherry). (E) Time course for mCherry protein expression from BL21(pDawn-mCherry) using 480 nm light. Light-dependent mCherry expression in BL21 cultures ($n = 3$ per time point) is significantly higher ($p < 0.001$) at all illumination times beyond 3 h (asterisks omitted at 4, 5, 6, and 8 h time points for clarity). All data are presented as mean ± SD.

pET26b(+) expression vector with a C-terminal His tag for protein purification (ClyA: BBa_K811000, ClyA-His: BBa_K811002). To verify that the synthesized product can lyse cancer cells, purified ClyA (0.3 nM) was added to cultured SKBR3 cells and cytotoxicity was determined using the commercial Cytotox-Fluor Cytotoxicity Assay (Promega). ClyA exhibited a significant 6-fold increase in cytotoxicity over the negative control vehicle (Figure 1A, $p < 0.001$) and a 1.2-fold increase in cytotoxicity over the digitonin positive control ($p < 0.001$), verifying that ClyA can potently lyse cancer cells. For more readily visualizing mammalian cell lysis by ClyA-producing bacteria, the remaining experiments were performed using classical blood agar assays.

dx.doi.org/10.1021/sb400174s | *ACS Synth. Biol.* 2014, 3, 944–948

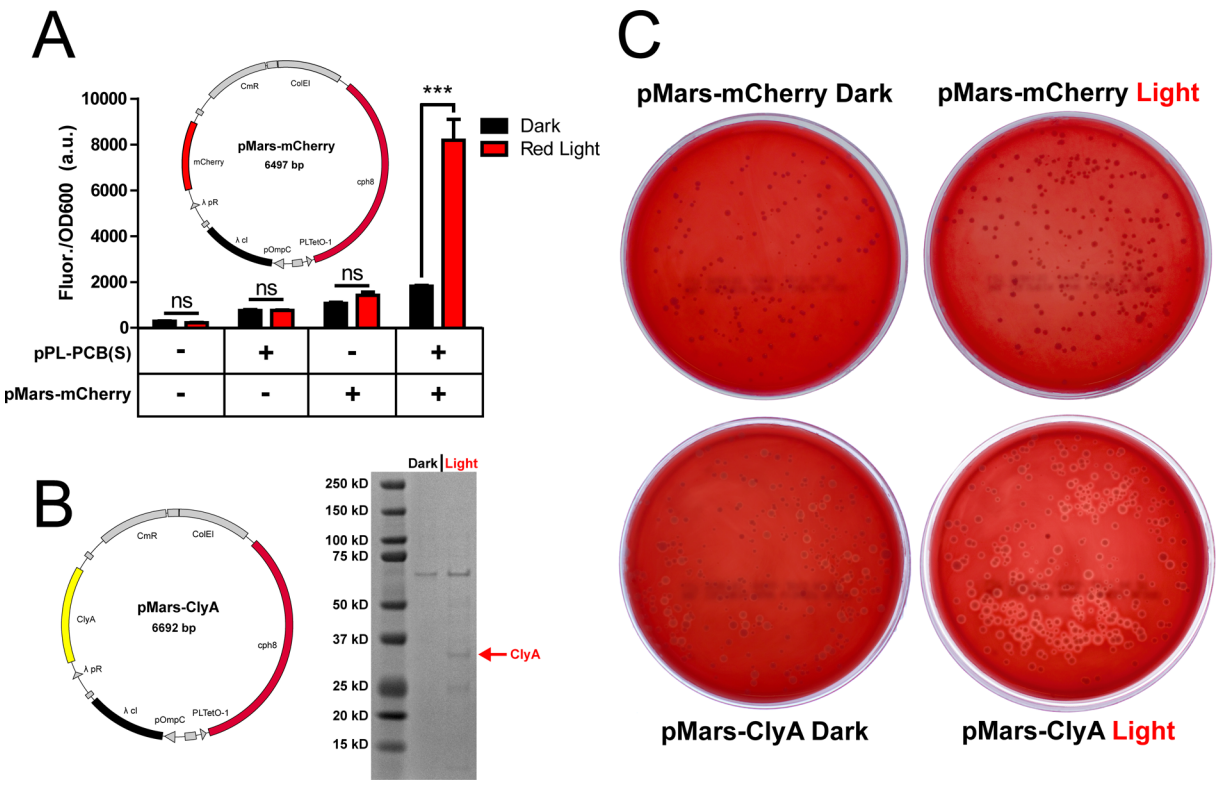


Figure 2. Red light-activated cytolysis in *E. coli* JT2 using pMars. (A) The red light-activated system was first tested with pMars-mCherry to ensure functioning of components. Specific, red light-induced expression of mCherry was measured by fluorescence ($p < 0.001$ between dark and light) and this effect was dependent on the presence of both the chromophore synthesis plasmid pPL-PCB(S) and pMars-mCherry ($p > 0.05$ between dark and light in first three conditions). (B) Red light-activated system with pMars-ClyA demonstrates weak light-dependent expression of the ClyA protein (~34 kDa). (C) JT2(pMars-ClyA) demonstrate red light-activated cytolysis on blood agar plates, while this effect is not observed for JT2(pMars-mCherry). All data are presented as mean ± SD.

The pDawn plasmid, a well-characterized optogenetic tool,[7] was used to achieve blue (480 nm) light-activated gene expression. To obtain light-dependent ClyA expression, the *clyA* gene was ligated into pDawn while adding an N-terminal His tag (His-ClyA: BBa_K811001), creating the plasmid pDawn-ClyA. *E. coli* BL21(DE3) cells were transformed with pDawn-ClyA and induced with blue light (or kept in the dark) at 25 °C for 24 h. ClyA was purified and a 34-kDa band was visible only in the blue light condition, verifying light-dependent production of ClyA (Figure 1B). To visualize light-activated cytolysis, we subsequently plated pDawn-ClyA-bearing bacteria onto blood agar plates. To further demonstrate efficacy in a clinically relevant strain, we also transformed pDawn-ClyA into *E. coli* Nissle 1917, a nonpathogenic and minimally immunogenic strain which is already used in clinical applications[8] and has tumor-targeting capabilities in mice.[9] By illuminating plates with blue light, we achieved inducible cytotoxicity with both BL21(DE3) and Nissle 1917, as evidenced by the clear zones of lysis surrounding only those colonies that were both transformed with pDawn-ClyA and illuminated with blue-light (Figures 1C,D). When bacteria were transformed instead with the pDawn plasmid containing the noncytotoxic protein mCherry, no light-dependent cytolysis was observed.

A key advantage of light-activated cytotoxicity is that the amount of protein produced can be finely tuned by controlling the illumination time. In a preliminary demonstration, we switched the output of our pDawn plasmid to mCherry and demonstrated control over total protein production by varying illumination time (Figure 1E). In the future, this tool could be calibrated based on the potency of ClyA to precisely produce the minimum amount of protein needed for cytolysis of a given tissue volume.

For eventual *in vivo* applications, the ideal light-activated therapeutic would be triggered by wavelengths longer than 480 nm because blue light is relatively poor at penetrating tissue. We thus sought to construct a new tool for red light-activated payload delivery. We developed a new plasmid backbone, pMars, which uses the well-characterized cph8[10] red-light sensor to activate gene expression with 650 nm light. A pMars-mCherry plasmid (red-light activated mCherry expression, Figure 2A) or a pMars-ClyA plasmid (red-light activated ClyA expression, Figure 2B) was transformed into *E. coli* JT2. The pPL-PCB(S) plasmid, which encodes the chromophore synthesis genes necessary to functionalize cph8,[10] was cotransformed with the above plasmids. Red light- and chromophore-dependent mCherry expression was demonstrated with pMars-mCherry (Figure 2A, $p < 0.001$) and red light-induced expression of ClyA was achieved with pMars-ClyA (Figure 2B). JT2 is a knockout strain that is not optimized for protein expression and purification and we did indeed observe lower purity of His-tagged ClyA recovered from JT2 (Figure 2B) compared to BL21(DE3) (Figure 1B), as evidenced by additional ~25-kDa and ~9-kDa light-dependent bands (hypothesized to be either cleavage-products of the 34-kDa ClyA protein or endogenous JT2 proteins that are copurified with ClyA) and a ~67-kDa constitutive band (hypothesized to be an endogenous JT2 protein that interacts with the

dx.doi.org/10.1021/sb400174s | *ACS Synth. Biol.* 2014, 3, 944−948

purification column). Nevertheless, the level of ClyA expression achieved with this strain was sufficient for eliciting red light-mediated cytotoxicity, with clear zones of lysis surrounding pMars-ClyA bearing colonies plated onto blood agar (Figure 2C). Given the natively low on/off ratio of the cph8 sensor,[10] successful light-induced cytolysis is likely due to the strong potency of the ClyA toxin (Figure 1A). In our experiments, the better performance of pDawn relative to pMars is due to the properties of the strains and circuits themselves, because no diffusion barriers were placed between the light sources and the bacterial colonies.

Engineered bacteria hold promise as potential therapeutic agents, and one of the factors that will determine the success of this approach is the choice of induction mechanism for therapeutic delivery. While other triggering methods have been explored previously;[2] here, we have introduced light-activated bacterial drug delivery, which is uniquely suited for achieving precise dosage control not only temporally but also spatially. Optogenetic control of cytotoxicity toward mammalian cells was demonstrated using two engineered strains of *E. coli*, including clinically relevant Nissle 1917. Blue or red light was used to successfully induce the desired cytolytic response, although further optimization of the red-light-activated system will be necessary to improve its dynamic range and reduce its leakiness, especially for *in vivo* applications. More generally, this approach could be used for light-activated expression of molecular payloads other than ClyA, including cytokines or other cytotoxic agents for specific cancer applications.[2]

MATERIALS AND METHODS

Plasmids and Strains. The pDawn and pPL-PCB(S) plasmids were provided by Andreas Möglich and J. Clark Lagarias, respectively. The plasmids pJT122 and pJT106b were purchased from Addgene (Plasmids 31396 and 31392, respectively). The *E. coli* strain JT2 was obtained from Jeff Tabor. BL21(DE3) strains transformed with pDawn-mCherry or pDawn-ClyA were maintained in 50 μg/mL kanamycin. JT2 strains transformed with pPL-PCB(S) and either pMars-mCherry or pMars-ClyA were maintained in 50 μg/mL kanamycin, 17 μg/mL chloramphenicol, and 100 μg/mL spectinomycin. Cultures were grown at 37 °C with shaking (250 rpm) unless otherwise specified. SKBR3 cells were obtained from Matthew Lazzara and the cells were routinely cultured at 37 °C in a humidified atmosphere with 5% CO_2.

Cloning. The Cytolysin A (*clyA*) gene from *E. coli* K12 (locus b1182) was codon optimized and synthesized (Integrated DNA Technologies). Standard restriction enzyme and ligase cloning was used to construct pET26b-ClyA (*Nde*I and *Xho*I restriction sites), pDawn-mCherry, and pDawn-ClyA (*Nde*I and *Bam*HI restriction sites). To construct pMars-mCherry and pMars-ClyA, the following DNA fragments from pJT122 (Addgene), pJT106b (Addgene), and pDawn were amplified by PCR. From pJT122, a 4.2 kb fragment was amplified using Primers 1 and 2 (Supporting Information Table 1). This backbone fragment included the cph8 gene, the kanamycin resistance gene, and the ColEI origin of replication and had *Xma*I and *Bgl*II restriction sites at each end. The pOmpC promoter and the cI repressor gene from pJT106b were amplified using Primers 3 and 4. The λ promoter and mCherry/ClyA from the pDawn plasmid were amplified using Primers 5 and 6. Using overlap assembly PCR with a 20 bp overhang, the two fragments were combined to create an insert that was then ligated into the backbone amplified from pJT122 with *Xma*I and *Bgl*II to create either pMars-mCherry (with the mCherry reporter gene) or pMars-ClyA (with the ClyA gene).

Protein Purification and Cytotoxicity Assay. Overnight cultures of BL21(DE3) transformed with pDawn-ClyA or JT2 cultures transformed with pMars-ClyA and pPL-PCB(S) were diluted and allowed to grow to an OD_{600} of ~0.8 and then induced with 1 mM IPTG, blue light (~480 nm and 37.1 μW/cm^2), or red light (~650 nm and 17.7 μW/cm^2) (similar red light power magnitudes have been previously used to induce cph8[10]) at 25 °C. Cultures were pelleted by centrifugation and sonicated. His-tagged proteins were column-purified using low-density nickel agarose beads (Gold Biotechnology). Samples were run on 1 mm 4−12% Bis-Tris SDS-PAGE gels and protein loading was standardized by the Bradford Assay. Cytotoxicity assays using the Cytotox-Fluor kit (Promega) were conducted according to the manufacturer's instructions with purified ClyA diluted in water. Emission values at 520 nm were normalized to calculate fold change over the negative control vehicle (water).

Light-Induction Time Courses. BL21(DE3) transformed with pDawn-mCherry or JT2 transformed with pMars-mCherry and pPL-PCB(S) were grown overnight in dark conditions and diluted into 50 mL cultures at an OD_{600} of ~0.1. Cultures were transferred to a shaker-incubator where they were illuminated continuously by blue or red light. For time course experiments, 5 mL of these cultures were transferred to dark conditions at the given illumination times. All cultures were assayed on fluorescent plate reader at the end of the 24 h period.

Blood Agar Assays. Overnight cultures of BL21(DE3) transformed with pDawn-ClyA or JT2 transformed with pMars-ClyA and pPL-PCB(S) were diluted 1:10^7 in LB and plated on Columbia agar plates seeded with 5% sheep erythrocytes (BD Diagnostics). Following 12 h of growth in the dark at 37 °C for colony formation, plates were switched to either light (blue ~480 nm, 37.1 μW/cm^2, or red ~650 nm, 17.7 μW/cm^2) or dark conditions at 25 °C for 24 h prior to imaging. Negative controls of pDawn-mCherry and pMars-mCherry were prepared in the same fashion. All blood agar experiments were repeated in at least 3 independent trials.

Statistics. Cytotoxicity results and pDawn-mCherry time points were compared using one-way ANOVA with Bonferroni posthoc comparisons. Red light-activated mCherry expression in pMars-mCherry was compared using two-way ANOVA with Bonferroni posthoc comparisons. Data are presented as mean ± standard deviation (SD).

ASSOCIATED CONTENT

Supporting Information

Table S1: Primers used to construct pMars plasmids. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Author Contributions

M.S.M. and A.V. conceived and developed the project idea in consultation with J.S.M. and C.A.S. M.S.M., A.V., P.Q., A.A., and J.Y.L. designed and performed experiments. M.S.M., A.V., J.S.M., and C.A.S. wrote the manuscript. J.S.M., M.G., and C.A.S. supervised all aspects of the project.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge Daphne Ng and Najaf Shah for helpful experimental discussions, Sevile Mannickarottu for providing laboratory space, and the iGEM competition for providing a platform for undergraduate synthetic biology research. The following materials were generously gifted: SKBR3 cells by Matthew Lazzara (University of Pennsylvania); the pDawn plasmid by Andreas Möglich (Humboldt University of Berlin); the pPL-PCB(S) plasmid by J. Clark Lagarias (University of California, Davis); and the strain JT2 by Jeff Tabor (Rice University). This work was supported by the University of Pennsylvania and a National Science Foundation CAREER Award (#CBET-1055231) to C.A.S. The 2012 Penn iGEM Team wiki can be found at http://2012.igem.org/Team:Penn, and the URL's for the BioBricks described in this work are as follows: http://parts.igem.org/Part:BBa_K811000, http://parts.igem.org/Part:BBa_K811001, http://parts.igem.org/Part:BBa_K811002.

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